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TITLE: Exploring the Role of BET Bromodomain Proteins in AR Transcriptional Regulation: A Perpetuating Cycle of JQ1 Resistance in CRPC Therapy

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13. SUPPLEMENTARY NOTES

14. ABSTRACT **During the inevitable progression to castration resistant stage, prostate cancer cells acquire resistance to anti-androgens. Paradoxically, despite the prolonged AR antagonist treatment, expression of AR is not abated in CRPCs, signifying AR as the *Achilles heel* to achieve realistic therapeutic benefit in CRPC patients. Recently, it has been reported that prostate cancer cells positive for AR-signaling and CRPC xenograft tumors display exquisite sensitivity to JQ1, a BET bromodomain inhibitor. Mechanistically, JQ1 binds the N-terminal BET bromodomain of BRD4 to disrupt its interaction with AR and prevent expression of DHT dependent AR target genes. Ironically, while JQ1 ablated AR target gene transcription, it significantly increased AR transcription and this increase was concomitant with increasing concentrations of JQ1 treatment. This incongruous observation suggests that in androgen-responsive PC cells, AR-BRD4 interaction may regulate AR transcription by a negative feedback loop. One potential mechanism is the presence of an AR transcriptional repressor that is directly regulated by the BRD4-AR complex, which maintains AR homeostasis. The hypothesis is that in cells treated with JQ1, inhibition of BRD4-AR signaling would prevent transcription of the AR repressor, consequently inducing AR transcription. Thus, greater the JQ1 mediated inhibition, greater is the upregulation of AR. Additionally, since only a fraction of the AR binding sites are co-enriched for BRD4 binding in androgen stimulated PC cells, JQ1 mediated inhibition will at the most have a modest effect on long-term CRPC treatments. It is this BRD4-independent AR target gene expression that will continue to promote CRPCs progression and metastasis. This proposal will explore novel molecular mechanisms by which prostate cancer cells develop resistance to BET bromodomain inhibitors. Further, it will explore the role of BCOR as an AR transcriptional repressor- to illuminate the reason for why JQ1 could be counterproductive for CRPC patients.**

15. SUBJECT TERMS **Androgen Receptor, BET Bromodomain proteins, Castration Resistant Prostate Cancer, JQ1, BRD4.**

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Table of Contents

Page

Exploring the Role of BET Bromodomain Proteins in AR Transcriptional Regulation: A Perpetuating Cycle of JQ1 Resistance in CRPC Therapy

1. Introduction

Androgen receptor (AR) plays a critical role in the onset and progression of prostate cancer (PC) by activating androgen-dependent and independent transcription programs, despite deprivation of its agonist, testosterone, an androgen. During the inevitable progression to Castration Resistant Prostate Cancer (CRPC), cells acquire resistance to anti-androgens, the most common therapeutic option currently used to treat advanced PCs. Paradoxically, despite the prolonged AR antagonist treatment, expression of AR is not abated, signifying AR as the *Achilles heel* to achieve realistic therapeutic benefit in CRPC patients. Recently, it has been reported that PC cell lines positive for AR-signaling and CRPC xenograft tumors formed by VCaP cells display exquisite sensitivity to JQ1 (IC_{50} 50nm), a BET bromodomain inhibitor (1). BET bromodomain containing proteins bind acetylated lysine residues in histone H3 and modulate transcription by recruiting histone modifying enzymes or chromatin interacting proteins, and can either activate or repress transcription depending on the context (2). Mechanistically, JQ1 binds the N-terminal BET bromodomain of BRD4 to disrupt its interaction with AR and prevent expression of DHT dependent AR target genes such as prostate-specific antigen (PSA) and ERG by inhibiting transcription (1). However, careful analysis of these data revealed that while JQ1 ablated AR target gene transcription, due to their co-dependency on BRD4 signaling, it significantly increased AR transcription and this increase was concomitant with increasing concentrations of JQ1 treatment (1). This incongruous observation suggests that in androgen-responsive PC cells, AR-BRD4 interaction may regulate AR transcription by a negative feedback loop. One potential mechanism is the presence of an AR transcriptional repressor that is directly regulated by the BRD4-AR complex, which maintains AR homeostasis. Consistent with this prospect, in cells treated with JQ1, inhibition of BRD4-AR signaling would prevent transcription of the AR repressor, consequently inducing AR transcription. Thus, greater the JQ1 mediated inhibition, greater is the upregulation of AR. Additionally, since only a fraction of the AR binding sites are co-enriched for BRD4 binding in androgen stimulated PC cells, JQ1 mediated inhibition will at the most have a modest effect on long-term CRPC treatments. It is this BRD4-independent AR target gene expression that will continue to promote CRPCs progression and metastasis. Not surprisingly, only 50% inhibition of castration resistant xenograft tumor growth was observed in mice treated for a month with 50mg/kg daily of the JQ1 inhibitor (1). Therefore, while trials with BET inhibitors e.g. GSK525762 (GlaxoSmithKline), CPI-0610 (Constellation Pharmaceuticals), TEN-010 (Tensha Therapeutics), and OTX-015 (Oncoethix) have been initiated for a variety of malignancies (and several other BET inhibitors are in pre-clinical development), their efficacy in PC may be limited due to its confounding effect on AR transcription itself.

Hypothesis/Rationale: Bioinformatics analysis of a large cohort of data on prostate cancer patients available at cBioportal revealed the expression of a transcriptional repressor, a BCL6 co-repressor (BCOR) complex, which positively correlates with AR expression in human primary adenocarcinomas (3). Our original hypothesis was that BCOR is an AR target gene; AR induces BCOR transcription, which in turn curtails AR levels, resulting in maintaining AR homeostasis in PC cells. However, in CRPCs treated with JQ1, the transcriptional repression of AR is relieved due to low BCOR expression; as a result, this robust increase in AR levels makes cells resistant to treatment with JQ1 and other BET bromodomain inhibitors. If BCOR was not

regulated by AR, as an alternative AR regulator, we also analyzed expression of HOXB13 in prostate cancer cells treated with the BET inhibitors.

2. Keywords

Androgen Receptor, BET Bromodomain proteins, Castration Resistant Prostate Cancer, JQ1, BRD4.

3. Accomplishments

Major Activities We performed several experiments to determine 1. the mechanism of action of the BET bromodomain inhibitor JQ1 in prostate cancer cells that has been previously reported to inhibit androgen dependent AR target gene expression 2. And whether CRPCs cells upregulate AR transcription to overcome the anti-proliferative effect of JQ1.

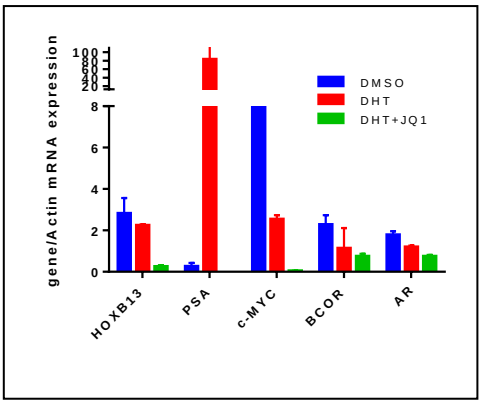

Fig 1. Analysis of AR, BCOR and HOXB13 mRNA expression in androgen stimulated and BET inhibitor treated PC cells. Relative quantitation of AR, BCOR and HOXB13 mRNA levels in VCaP cells treated with 2.5 uM JQ1 for 16h. DHT stimulation 10 nM for 6h. c-myc-positive control for BET inhibition. JQ1 is also known to suppress AR target gene expression such as the Prostate specific antigen, PSA. Actin is used as anormalization control. Error bars denote 95% confidence intervals.

1. Objectives

(1) Evaluate BCOR expression in androgen and JQ1 treated prostate cancer cells and assess its correlation with AR mRNA expression. **Alternatively,** if we do not see differences in BCOR mRNA expression in androgen-dependent manner or loss of expression in JQ1 treated cells, we will examine a second transcriptional co-repressor HOXB13, whose expression was found to be co-modulated with AR expression in prostate adenocarcinomas (4).

(2) Determine whether BRD4-AR complex is recruited to the HOXB13 genomic regions.

(3) Assess whether JQ1 alleviates HOXB13 mediated transcriptional repression of AR *in vivo* to protect CRPCs from JQ1 treatment.

2. Results

1. **Evaluate BCOR and HOXB13 gene expression in androgen and JQ1 treated prostate cancer cells and assess its correlation with AR mRNA expression.** To determine whether DHT mediated AR activation leads to increase in BCOR expression which in turn causes AR mRNA suppression, qRT-PCR was performed. In brief, VCaP (**Figure 1**) was grown in androgen deprived media

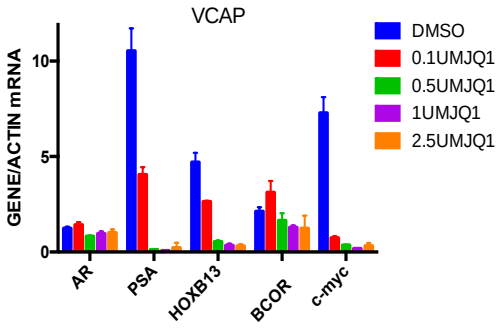

Fig 2. HOXB13 mRNA but not AR expression is suppressed by BET inhibitor JQ1 in VCaP cells. Relative quantitation of AR, BCOR and HOXB13 mRNA levels in VCaP cells treated with JQ1 for 16h at various doses (0.1 to 2.5 uM). c-myc and PSA are used as positive controls to demonstrate JQ1 effect. Error bars denote 95% confidence intervals.

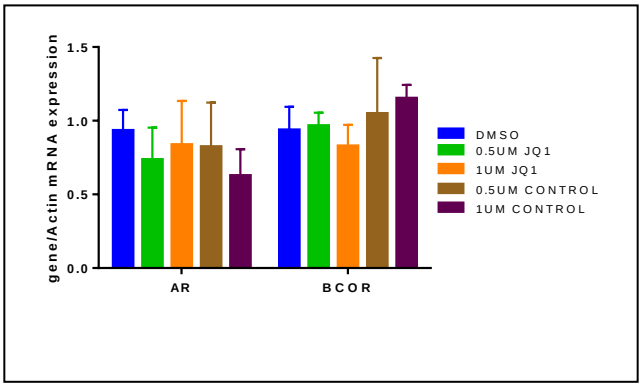

Fig 3.BET inhibitors do not modulate the expression of AR and BCOR expression in castration resistant 22RV1 cell line. Relative quantitation of AR, BCOR and Actin mRNA levels in 22RV1 cells treated with JQ1 for 16h at various doses (0.5 to 1 uM). Error bars denote 95% confidence intervals.

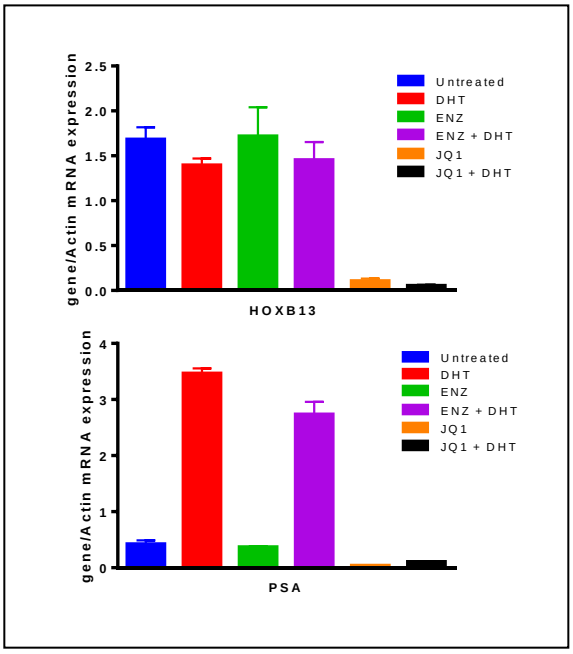

Fig 4. HOXB13 mRNA expression is androgen independent, resistant to enzalutamide but affected by BRD4 inhibitor, JQ1. Relative quantitation of HOXB13 and PSA mRNA levels in LNCaP cells treated with JQ1 or Enzalutamide (16h). Actin is used as a normalization control. Error bars denote 95% confidence intervals.

(charcoal stripped FBS containing media) for 2 days and then cells were treated with DMSO (control) or JQ1 (2.5 um) for 24 hours. Subsequently, the prostate cancer cells were treated with DHT (10 nm, 6 hrs) or untreated as control, and total RNA was isolated. qRT-PCR was performed to evaluate mRNA levels of BCOR, AR, HOXB13, and actin levels and expression of the transcripts relative to Actin mRNA were determined. In addition, mRNA expression of PSA, an AR-target gene, and c-MYC a BRD4 regulated gene was also determined. In contrast to the expression of the AR target gene PSA (positive control) which showed a significant increase following the addition of DHT, BCOR expression was found to be decreased (**Figures 1**). Both PSA and c-myc expression was inhibited in BET inhibitor treated cells consistent with previous reports (**Figures 1, 2 and 4**).

As previously reported in literature, AR mRNA levels were not induced by DHT but instead a modest in decrease in AR levels was observed in VCaP cells (**Figure 1**) (5). Further, in contrast to published report (1), AR mRNA levels were not increased following JQ1 treatment (**Figures 1-3)**.

We also analyzed the expression of HOXB13 in VCaP and LNCaP cells (**Figures 1, 2 and 4**) which we had proposed as an alternative to

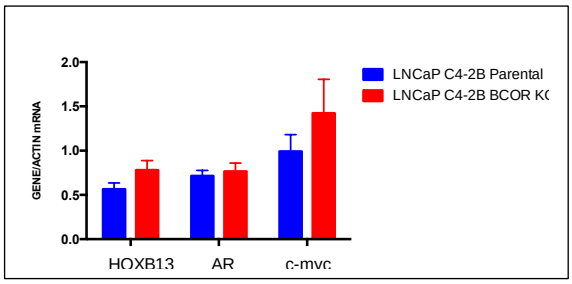

Fig 5.Ablation of BCOR does impact AR or HOXb13 levels. LNCaP C4-2B cells were transfected with BCOR CRISP/Cas9 gene editing constructs. Relative quantitation of HOXB13, AR and c-myc mRNA levels in were examined. Actin is used as a normalization control. Error bars denote 95% confidence intervals.

BCOR as a target gene and has been reported to function either as an AR activator or repressor in a context dependent manner (6). In contrast to BCOR, we found that DHT had no effect on HOXB13 expression, while treatment with JQ1 completely suppressed HOXB13 expression (**Figure 1, 2 and 4**). BCOR also did not regulate HOXB13 expression (**Figure 5**). Conversely, knockdown of BCOR had no effect on AR transcription (**Figure 5**). Combined these results suggest that BCOR may not negatively regulate AR transcription in prostate cancer cells.

HOXB13 is known to function as an AR co-activator for genes that contain homeobox response elements (HOXRE), suppression of HOXB13 gene expression by BET bromodomain inhibitors may inhibit androgen-independent AR/HOXB13 transcriptional programs as well as HOXB13 regulated transcriptional programs essential for prostate cancer survival. Indeed knockdown of HOXB13 by RNAi had a significant impact on the growth of LNCaP cells which was comparable to inhibition of BRD4 (**Figure 6**). **<u>These results suggest for the first time that HOXB13 is epigenetically regulated by the BET bromodomain proteins. Importantly, HOXB13 is a novel therapeutic target of BET bromodomain inhibitors in prostate cancers and may in part underlie the therapeutic efficacy of JQ1 like inhibitors in CRPC.</u>**

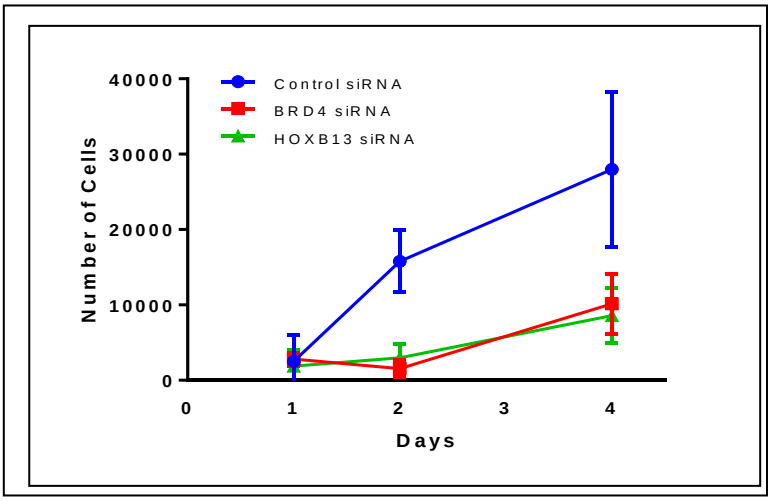

Fig 6.Effect of HOXB13, BRD4 knockdown on proliferation pf LNCaP cells. LNCaP cells were transfected with various siRNA as indicated in the legend. Cell proliferation was measured over a 96 hour period. Viable cells at the end of 96 h were counted by trypan blue dye exclusion assay. N=8 for each condition.

We pursued subsequent experiments as outlined below to determine the significance of BRD4 mediated epigenetic regulation of HOXB13 expression.

2.CHIP-PCR to determine whether BRD4-AR complex is recruited to the HOXB13 genomic regions. BRD4 recognizes diacetylated histone

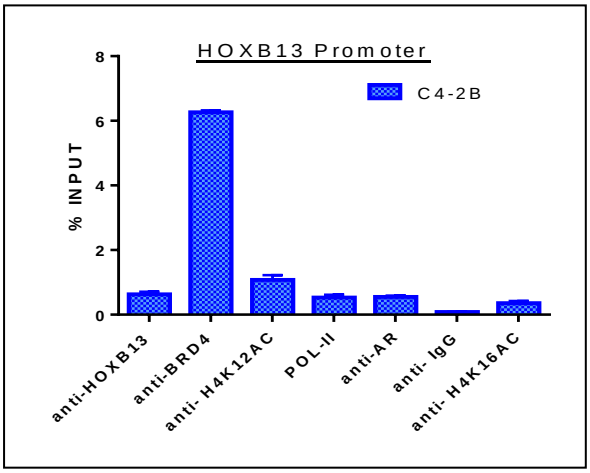

Fig 7. HOXB13 is epigenetically regulated by the BET bromodomain protein BRD4 in CRPC. A-B. Schematic of HOXB13 genomic region indicating the potential BRD4 binding sites. TSS-Transcription Start Site. CHIP-qPCR was performed with extracts prepared from the metastatic PC cell line C4-2B with various antibodies (anti-BRD4, anti-H4K12Ac, anti-AR, anti-H4K16Ac and IgG) and analyzed for HOXB13 promoter and enhancer region. Set 14 primer is 1kb from TSS.

residues *i.e* H4K5ac/8ac, H4K12ac/16ac, H4K1ac/20ac, in the chromatin and is particularly enriched at enhancer and super-enhancer regions, which strongly stimulates the expression of some oncogenes in cancer (7). Mechanistically, BRD4 can promote transcription by recruiting the mediator complex to the acetylated chromatin regions at distal enhancers (8). BRD4 can also recruit the positive transcription elongation factor B (PTEFB; (CDK9)/cyclin T complex) to the acetylated promoter regions, leading to phosphorylation of CTD of RNA polymerase II (RNA Pol II) to promote transcription. We detected a putative BRD4 binding site in the HOXB13 promoter/enhancer region near the transcription start site that is sensitive to the novel BET inhibitors MA4-22-2 and SG3-179 treatments but not JQ1 or Enzalutamide (**Figure 7 and Figure 8**). Very low levels of AR and HOXB13 recruitment to the HOXB13 promoter were observed (**Figure 7)**. In future we will perform CHIP-seq analysis to identify the potential BRD4 binding sites in HOXB13 promoter region in an unbiased manner that are affected by JQ1 treatment.

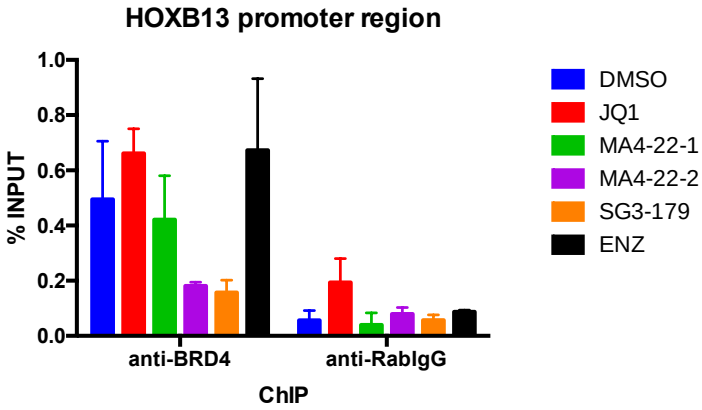

Fig 8. BRD4 recruitment to HOXB13 regulatory region. The red bar shows putative BRD4 binding sites identified in the HOXB13 promoter regions. MA4-22-1, MA4-22-2 and SG3-179 arenovel potent inhibitors of BRD4 recruitment. ChIP was performed with anti-BRD4 polyclonal Abs and anti-rabbit IgG(control) followedby qPCR with Primer 8 in HOXB13 promoter region.

3. **Assess the ability of HOXB13 to promote CRPC growth and effect of JQ1 on inhibition of CRPC growth.** Two complementary approaches were used to test the role of JQ1 or HOXB13 knockdown in inhibiting CRPC growth.

VCaP cells HOXB13 KO cells were generated by CRISPR/Cas9 gene editing technology. $1X10^6$ VCaP cells (parental or HOXb13 KO cells) were injected subcutaneously in immunocompromised male mice. None of the VCaP HOXB13 pKO cells formed tumors (**Figure 9 top**

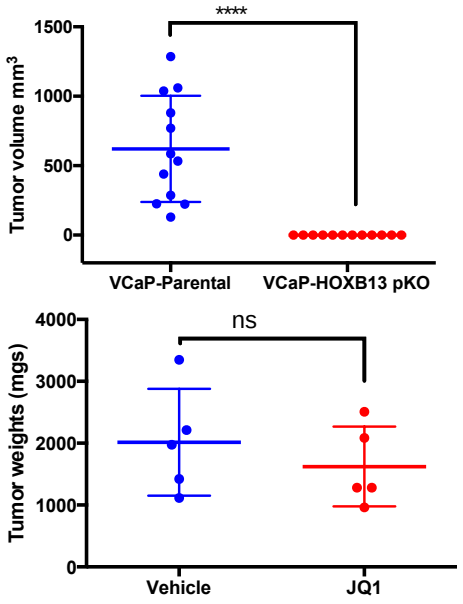

Fig 9 A. Impact of HOXB13 gene deletion on VCaP xenograft tumor growth. Intact SCID male mice were injected subcutaneously with parental or HOXB13 pKO cells and growth was monitored over a 6 week period. B. Male SCID mice were injected subcutaneously with VCaP cells. Once mice formed palpable tumors, mice were castrated. Mice were injected with vehicle or JQ1 (30 mg/kg of body) or vehicle. Final tumor weights are shown. Tumor growth was attenuated but not completely regressed in JQ1 treated mice.

panel) suggesting that HOXB13 is absolutely critical for CRPC growth

SCID mice were castrated after palpable VCaP tumors were formed. Mice were injected with JQ1 (30 mg/Kg) every alternate day for 4 weeks (DMSO as vehicle control). JQ1 was able to partially inhibit the growth of VCaP cells (**Figure 9 bottom panel)**.

We could not test 50mg/kg concentration of JQ1 as at higher concentration the compound precipitated out of the aqueous phase.

Overall, this study reveals novel molecular mechanisms by which the BET bromodomain inhibitors may impact the growth of CRPC cells. Significantly, it provides evidence for the role of prostate-specific transcription factor, HOXB13, as an androgen independent driver of transcriptional programs in PC cells whose expression can be targeted with the BET bromodomain inhibitors. Moreover, HOXB13 expression is not affected by the anti-androgen Enzalutamide, suggesting that HOXB13 driven transcription programs may underlie resistance to anti-androgen therapies.

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- **What opportunities for training and professional development has the project provided?**

Seminars:

1. Tumor Biology Retreat, Tampa Palms Club House, Tampa, Florida, December 2016 **HOXB13: Validation of Targets in Human Prostate Cancers and Clinical Translatability Studies**
2. Tumor Biology, Research in Progress Seminar Series October 2016**: HOXB13 as a therapeutic vulnerability in Castration resistant prostate cancer**

3. Tumor Biology Retreat, Tampa Palms Club House, Tampa, Florida, December 2015
   Recalcitrant Cancers, Cancer Stem Cells and Targeted Therapies
4. Moffitt Cancer Center, Tumor Biology Seminar Series, Tampa, Florida, November, 2015
   Exiting the Comfort Zone: Tackling the Complexity of Castration Resistant Prostate Cancer

- **How were the results disseminated to communities of interest?**
  - **Participated and spoke at the Florida Prostate Cancer Symposium**. "Targeting HOXB13, a PC Risk Gene in Metastatic Prostate Cancers with Novel Epigenetic Inhibitors. May19-20, 2016
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - *"Nothing to Report."*

4. IMPACT:

Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

- **What was the impact on the development of the principal discipline(s) of the project?**
  HOXB13, a transcription factor, is highly expressed in prostate and is linked to high Gleason grade, positive lymph node status, high pre-operative PSA levels and early PSA recurrence in primary prostate cancers. The results from this study indicate that HOXB13 drives castration resistant prostate cancer growth independent of the Androgen Receptor. Moreover, HOXB13 positive cancers can be targeted with BET bromodomain inhibitors.

- **What was the impact on other disciplines?**
  HOXB13 gene expression may be targeted in other cancers where it is aberrantly expressed, such as Estrogen receptor positive tamoxifen resistant breast cancers with BET bromodomain inhibitors.
- **What was the impact on technology transfer?**
  - *"Nothing to Report."*
- **What was the impact on society beyond science and technology?**
  - *"Nothing to Report."*

5. **CHANGES/PROBLEMS:** "*Nothing to Report, "***Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents** *"Nothing to Report":*

a. **Significant changes in use or care of human subjects** *"Nothing to Report":*

b. **Significant changes in use or care of vertebrate animals.** *Nothing to Report."*

c. **Significant changes in use of biohazards and/or select agent** *"Nothing to Report."*

6. PRODUCTS

Publications, conference papers, and presentations

Report only the major publication(s) resulting from the work under this award.

i. **Journal publications**,

ii. 1. **Epigenetic Reprogramming of the Androgen receptor in CRPC** under review in *Cancer Cell*; acknowledgement of federal support (yes).

iii. 2.**BRD4 Mediated Epigenetic Regulation of HOXB13 Transcriptional Networks Promotes Castration Resistant Prostate Cancer Growth** (Manuscript under preparation); *acknowledgement of federal support (yes).*

iv. **Books or other non-periodical, one-time publications.**

Nothing to report

v. **Other publications, conference papers, and presentations.**

vi. **Florida Prostate Cancer Symposium**. "Targeting HOXB13, a PC Risk Gene in Metastatic Prostate Cancers with Novel Epigenetic Inhibitors. May19-20, 2016

vii. *Seminars:*
Tumor Biology Retreat, Tampa Palms Club House, Tampa, Florida, December 2016
HOXB13: Validation of Targets in Human Prostate Cancers and Clinical Translatability Studies

Tumor Biology, Research in Progress Seminar Series October 2016**: HOXB13 as a therapeutic vulnerability in Castration resistant prostate cancer**
Tumor Biology Retreat, Tampa Palms Club House, Tampa, Florida, December 2015
Recalcitrant Cancers, Cancer Stem Cells and Targeted Therapies
Moffitt Cancer Center, Tumor Biology Seminar Series, Tampa, Florida, November, 2015

Exiting the Comfort Zone: Tackling the Complexity of Castration Resistant Prostate Cancer

d. **Website(s) or other Internet site(s)**

nothing to report

e. **Technologies or techniques**

nothing to report

f. **Inventions, patent applications, and/or licenses**

nothing to report

g. **Other Products**

nothing to report.

7…PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	***Kiran Mahajan***
Project Role:	*Assistant Professor*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Mahajan performed cell culture studies, bioinformatics analysis and analyses of data and writing of report.*
Funding Support:	*NA.*
Funding Support:	*NA*
Name:	***Niveditha Nerlakanti***
Project Role:	*Research Associate*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*12*
Contribution to Project:	*Niveditha performed western blotting, quantitative RT-PCR and animal experiments*
Funding Support:	*NA*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

"Nothing to Report."

What other organizations were involved as partners?

nothing to report

8…SPECIAL REPORTING REQUIREMENTS

nothing to report

9..APPENDICES:

nothing to report